Peer Review File

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Reviewer Comments

Introduction

Comment 1:

Lines 50-51: Replace: “The main bioactive substances in teas are tea catechins, tea polyphenols, tea polysaccharides and so on” to “Among the main bioactive substances in teas is worth to mention the catechins, polyphenols, caffeine, and polysaccharides”.

Reply 1:

Thanks for your help. We have modified our text as advised (see lines 59-60)

Changes in the text:

Among the main bioactive substances in teas is worth to mention the catechins, polyphenols, caffeine, and polysaccharides.

Comment 2:

Lines 51-53: Rephrase the sentence: “According to their degree of fermentation, teas are divided into six categories as follows: green tea, yellow tea, white tea, oolong tea, black tea, and dark tea. Among the four teas selected in this research, Ti Kuan Yin tea and Pu’er tea are types of semifermented oolong tea and whole-fermented dark tea, respectively” to “According to their degree of fermentation, teas are divided into six categories as follows: green tea, yellow tea, white tea, oolong tea, black tea, and dark tea. The green tea is considered unfermented, the oolong semifermented, whereas the dark tea is whole-fermented. (REFERENCE)”

Please include the following reference: Effect of fermentation conditions and plucking standards of teas leaves on the chemical components and sensory quality of fermented juices. Journal of Chemistry, 2018. DOI: 10.1155/2018/4312875

Reply 2:

Thank you very much for your guidance. We have modified our text as advised and added a reference (see lines 60-63)

Changes in the text:

According to their degree of fermentation, teas are divided into six categories as follows: green tea, yellow tea, white tea, oolong tea, black tea, and dark tea. The green tea is considered unfermented, the oolong semifermented, whereas the dark tea is whole-fermented (10).

10. Tang P, Shen DY, Xu YQ, et al. Effect of Fermentation Conditions and Plucking Standards of Tea Leaves on the Chemical Components and Sensory Quality of Fermented Juice. Journal of Chemistry 2018:1-7.

Comment 3:

Lines 68: please rephrase the sentence: "…and most of them have used biological methods." I would say that have used animal models.

Reply 3:

Thank you for your careful review. We apologize for the inaccuracy in this manuscript and the confusion that it caused in your reading. (see lines 79-80).

Changes in the text:

Previous reports have mainly focused on the effects of tea polyphenols and catechins on CYP450 isoforms (16,17,18), and most of them have used animal models.

Comment 4:

Lines 69-71: I suggested the authors inform that they used an in vitro approach as an attempt to minimize the use of animals in preliminary and screening investigations of tea—drug interactions.

Reply 4:

Thank you very much for your professional guidance. We have modified our text as advised (see lines 84-85).

Changes in the text:

Moreover, the in vitro approach was used as an attempt to minimize the use of animals in preliminary and screening investigations of tea-drug interactions.

Methods

Comment 5: Replace "Chemical and reagents" to Chemical and materials

Reply 5: Thanks for your help. We have modified our text as advised (see line 92).

Changes in the text: Chemicals and materials

Comment 6:

Lines 83-85: Replace: "Green tea, Pu'er tea and Ti Kuan Yin tea were provided by Yi Jiangnan Tea Industry Co. Ltd. (Hangzhou, China). Black tea was procured from Ming Tea Industry Co., Ltd. (Wuhan, China)" to "The Green tea and two oolong teas (Pu'er and Ti Kuan Yin) were provided by Yi Jiangnan Tea Industry Co. Ltd. (Hangzhou, China). The Black tea was procured from Ming Tea Industry Co., Ltd. (Wuhan, China) "

Reply 6:

Thanks for your help. We have modified our text as advised (see lines 101-103).

Changes in the text:

The green tea, oolong tea (Ti Kuan Yin) and dark tea (Pu'er) were provided by Yi Jiangnan Tea Industry Co. Ltd. (Hangzhou, China). The Black tea was procured from Ming Tea Industry Co. Ltd. (Wuhan, China).

Comment 7:

Please include: the chromatographic columns in case the authors decide to discuss method development.

Reply 7:

Thanks for your suggestion. We have modified our text as advised (see lines 98-99).

Changes in the text:

The chromatographic column was an Intersil ODS-2 column (5 μm, 4.6×150 mm) (Phenomenex, USA)

Selectivity and specificity

Comment 8:

Lines 170-173: One major question aroused when reading this section: How did the authors evaluate if the components of the tea samples are not affecting the selectivity and specificity as well as if the tea sample has matrix effect?

Reply 8: Thank you very much for your professional advice. In view of the advantages of LC–MS/MS, including high selectivity and specificity, the cocktail approach has been developed for monitoring several CYP activities in a single experiment. As the tea extract was regarded as the object of our study, the results of evaluation included multiple components of tea. As long as the chemical components in the tea extract interacted with CYPs, the concentrations of the probe drugs changed. LC–MS/MS directly determined the concentrations of the probe drugs, which could indirectly infer the inhibitory effect of tea extract with multiple components (see lines 329-334). In addition, the selectivity and lower limits of quantification were determined in the section "Selectivity and specificity", and the results indicated that the selectivity and specificity of the method met the requirements, as shown in Figure 2 and Table 2.

Furthermore, the matrix effect was estimated by comparing the peak area of the matrix-matched sample to that of a standard solution at an equivalent concentration for low, intermediate and high concentration levels. The method for the matrix effect has been modified in the section “Matrix effect and extraction recovery” (see lines 225-228). The matrix effects for analytes were in the range of 96.46-113.62%, which met the requirements of the measurement. The results are shown in the “Validation of the analytical method” section (see lines 284-286).

Changes in the text:

LC–MS/MS Evaluation

In view of the advantages of LC–MS/MS, including high selectivity and specificity, the cocktail approach has been developed for monitoring several CYP activities in a single experiment. The LC–MS/MS method directly determined the concentration of the probe drugs, which could indirectly infer the inhibitory effect of tea extract with multiple components.…

Matrix effect and extraction recovery

… The matrix effect was estimated by comparing the peak area of the matrix-matched sample to that of a standard solution at an equivalent concentration for low, intermediate and high concentration levels. Experiments were performed using six replicates at three concentrations (QC-L, QC-M and QC-H).

Validation of the analytical method

…As shown in Table 4, the extraction recoveries of all analytes were in the range of 71.31-95.17%. The matrix effects for most analytes were in the range of 96.46-113.62% at low, intermediate and high concentration levels.…

Results

Comment 9:

I suggest to the authors move the Enzyme kinetic profile of the probe cocktail substrates section after the Validation of the analytical method. It will be easier for the reader. Frist get the results of the analytical method and then the information about the inhibitory assay.

Reply 9:

Thanks for your suggestion. We have adjusted the order as advised (see lines 272-294).

Changes in the text:

Validation of the analytical method

Typical chromatograms of a blank RLM sample (A), a blank RLM sample spiked with drugs at the LLOQs and the IS (B), and an RLM sample incubated with the substrate cocktail and active RLMs (C) are shown in Figure 2. No significant influence of endogenous substances on the detection of analytes was observed. Calibration curves for each drug were linear over the determined concentration ranges, with correlation coefficient (R^2) values greater than 0.996. The standard curve, correlation coefficient, calibration range and LLOQs are presented in Table 2. The obtained intraday/interday accuracy and precision data were within the acceptable range for all of the analytes and are summarized in Table 3. The intraday accuracy ranged from -3.98% to 10.42%, with precisions ranging from 3.53% to 12.91%. Moreover, the interday accuracy ranged between -3.49% and 11.64%, while the precision ranged between 4.39% and 11.30%. The results suggested that the method was suitably accurate and precise. As shown in Table 4, the extraction recoveries of all analytes were in the range of 71.31-95.17%. The matrix effects for most analytes were in the range of 96.46-113.62% at low, intermediate and high concentration levels. The stability of the probes was satisfactory since the QC concentrations at the end of each condition remained within ± 15% of the theoretical values (MT: -2.60% - 3.62%; OM: 1.61% - 5.06%; PNT: 0.27% - 12.76%; TOL -10.00% - 4.13%; and T: 1.09% - 9.94%).

Enzyme kinetic profile of the probe cocktail substrates

To optimize the concentration of the probe substrates, an enzyme kinetic study was conducted. Kinetic profiles of five probe reactions are represented in Figure 1. As shown in Figure 1, the K_m values of MT, OMP, PNT, TOL and T were 4.921 μM, 9.662

μM, 33.02 μM, 157.1 μM and 44.05 μM, respectively.

Comment 10:

Table 5 to too big. In fact, I suggest including the results within the text and deleting the table. For example, “The stability of the probes was satisfactory since the QC concentrations at the end of each condition remained within ± 15% of the theoretical values (MT: -0.14% - 3.62%; OM: 1.61% - 13.15%; PNT: 0.27% - 14.78%; TOL -10.00% - 14.18%; T: 1.09% - 11.17%).

Reply 10:

Thanks for your suggestion. We have modified our text as advised (see lines 285-289), and Table 5 has been deleted.

Changes in the text:

The stability of the probes was satisfactory since the QC concentrations at the end of each condition remained within ± 15% of the theoretical values (MT: -2.60% - 3.62%; OM: 1.61% - 5.06%; PNT: 0.27% - 12.76%; TOL -10.00% - 4.13%; and T: 1.09% - 9.94%).

Table 5 has been deleted.

Comment 11:

I would suggest removing table 6, since the dose-response curves were presented (Figure 3), within each figure present the IC50, the confidence interval, and Emax% values there is no need to present St error since the data are log-transformed. The Graphpad guidance explains the theoretical approach. For example, check the references doi: 10.1002/jps.20975; doi:10.1038/clpt.2010.119

For the black tea, the dose-response is not adequate

Reply 11:

Thank you for your suggestion and for providing extensive guidance. We have studied the references you mentioned, which give us important and valuable help. Table 6 has been deleted, and the references have been added. For black tea, the dose response was indeed inadequate in all of the concentration ranges. It was reported that there is a significant inhibitory effect of drugs on CYP450 when the ReA% is less than 10%. Thus, black tea inhibited CYP1A2 in the concentration range of 300-700 μg mL^{-1}. Furthermore, Liu KH et al. proposed that strong inhibition appeared with IC_{50} <1000 μg mL^{-1}; otherwise, there was a weak inhibitory effect on the enzyme when IC_{50}> 1000 μg mL^{-1}. Consequently, black tea had a fairly weak inhibitory effect on CYP1A2 (see lines 299-300).

Changes in the text:

Table 6 has been deleted.

…aqueous extracts of black tea with intermediate concentrations only had a fairly weak inhibitory effect on CYP1A2, with an IC_{50} value of 840314 μg mL^{-1} …

Reference:

de Castro WV, Mertens-Talcott S, Derendorf H, et al. Grapefruit juice-drug interactions: Grapefruit juice and its components inhibit P-glycoprotein (ABCB1) mediated transport of talinolol in Caco-2 cells. J Pharm Sci 2007;96:2808-17.

Templeton I, Peng CC, Thummel KE, et al. Accurate prediction of dose-dependent CYP3A4 inhibition by itraconazole and its metabolites from in vitro inhibition data. Clin Pharmacol Ther 2010;88:499-505

Comment 12:

The supplementary material is not configurated adequately. The authors should check all figures!

Reply 12: Thank you for your suggestion. We have modified the format of all figures in the manuscript and supplementary material, and added figures about the lower limit of quantification of the five probe drugs in rat liver microsomes determined by HPLC–MS/MS in the supplementary material, as shown in Figure 9.

Changes in the text:

Manuscript: Figure1-3

supplementary material: Figure1-9

Comment 13:

Figure 7, keep only one decimal algorism!

Reply 13: Thank you for your suggestion. The axis has been modified with one decimal algorithm in Figure 7 and Figure 8.

Changes in the text:

supplementary material: Figure7, 8.

Discussion

Comment 14:

Lines 261-262: Rephrase the sentence “According to the processing technique, teas can be divided into green tea, black tea, oolong tea (Ti Kuan Yin tea), Pu'er tea and so on”. Avoid the term “so on”.

Reply 14:

Thank you for your suggestion. We have modified our text as advised (see lines 307-308).

Changes in the text:

According to the processing technique, teas can be divided into green tea, black tea, oolong tea (Ti Kuan Yin tea) and Pu'er tea.

LC–MS/MS method development

Comment 15:

1) The authors discussed method development but such information is missing under the material and methods section!

Within the material and methods section, the authors informed only the final conditions. I would suggest removing all information on method development under the discussion section! Or add another subsection under material and methods informing all steps of method validation, including the columns!

In my opinion information on the method, development is not relevant in this study.

2) Similarly, no need to discuss sample preparation

3) All this section could be deleted.

4) Authors should discuss firstly the LCMSMS results and afterward the inhibition studies!

Reply 15:

Thank you very much for your valuable comments and advice. We have deleted this part and added a discussion about LC–MS/MS before the inhibition studies (see lines 329-342).

Changes in the text:

Evaluation of LC–MS/MS

In view of the advantages of LC–MS/MS, including high selectivity and specificity, the cocktail approach has been developed for monitoring several CYP activities in a single experiment. The LC–MS/MS method directly determined the concentration of the probe drugs, which could indirectly infer the inhibitory effect of tea extract with multiple components. To ensure reliability during normal use, the analytical method was validated by a series of experiments, including the specificity, linearity, sensitivity, matrix effects, recovery, precision, accuracy, and stability. The results met the requirements of measurement. This demonstrated that the analytical method was suitable for its intended purpose and was accurate, specific and precise over the specified range that an analyte would be analysed. The results showed that the IC50 values of green tea were 652.2 μg mL^{-1} on CYP1A2 and 953.6 μg mL^{-1} on CYP2C6, the IC50 value of Pu'er tea tea was 738.7 μg mL^{-1} on CYP2C6, the IC50 value of Ti Kuan Yin tea was 1304.0 μg mL^{-1} on CYP1A2, and the IC50 value of black tea was 840314.0 μg mL^{-1} on CYP1A2, which were reliable.

Optimization of incubation conditions

Comment 16:

Lines 313: The sentence: "Moreover, no more than 10-30% of the probe substrates or inhibition depletion was suggested (34)" is meanness? Please rephrase! The same sentence is repeated on lines 323-324!

This section is quite cloudy to understand, authors should revise the English, should be more concise.

Reply 16:

Thank you very much for your professional advice. We have modified this section (see lines 343-363).

Changes in the text:

Optimization of the incubation conditions

In our experiment, different protein concentrations were evaluated. It was reported that the concentration of protein in the incubation system might have a major effect on the estimation of the inhibitory potency of inhibitors and contribute to variations among laboratories (31-33). Thus, the lowest possible microsomal protein concentration was used to reduce the probability of nonspecific binding of substrates to microsomal proteins for in vitro studies of metabolic inhibition (34). The depletion of all five probe drugs was linear with increasing protein concentration (0.1-0.8 mg mL^{-1}) at an incubation time of 30 min. Considering protein binding as well as reaction rates, a 0.5 mg mL^{-1} protein concentration was used.

For optimization of the incubation time, the linear relationship between the incubation time and the depletion of all five probe drugs was evaluated within 5-60 min. The results showed that the depletion of all five probe drugs was linear with increasing incubation time from 5 to 30 min (35,36), and the depletion of each probe drug was less than 30%, which was recommended. However, the depletion of testosterone decreased after 30 min, which indicated that there would be inhibition of testosterone if the incubation time was too long. Moreover, due to the instability of enzymes in vitro, the reaction time should be appropriately short. Therefore, 30 min was chosen as the incubation time.

In addition, the concentration of organic solvents in the incubation mixture has been reported previously and was indicated to be kept below 0.5-1% (v/v) to maintain the activities of CYPs (at least 80% activity) (37,38). The methanol concentration was set to < 1% (v/v) in our incubation system.

Inhibition of four types of tea on the activity of CYP450 isoforms

Comment 17:

Authors should compare the results they found with results obtained from other authors investigating inhibitory effects of tea on CYP activity. What are the putative components able to cause such inhibition?

Reply 17: Thank you for giving us more extensive and in-depth research guidance. We have added this part (see lines 387-410).

Changes in the text:

Due to interspecies differences, in vitro studies have suggested that tea extract (mainly green tea) inhibits the activities of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4 to varying degrees (43,44). Among them, rat models have reduced enzyme activities of CYP2C, CYP2E1 and CYP3A with the treatment of green tea and black tea (45,46) and increased the activity of CYP3A by drinking oolong tea

(47). In view of regional differences and slight changes in the processing technique, the results obtained may be different from those of previous studies. In our study, four teas inhibited different CYPs: green tea inhibited CYP1A2 and CYP2C6, black tea inhibited CYP1A2, Pu'er tea inhibited CYP2C6 (oolong tea), and Ti Kuan Yin inhibited CYP1A2 (dark tea). In addition, the chemical components of tea mainly consist of phenols, alkaloids and amino acids, some of which have been linked to health benefits and give tea its bitter taste and astringent quality (48). Most phenolic compounds found in tea are polyphenols, which are represented by catechins. Catechins and their derivatives have significant inhibitory activities on CYPs, of which gallated catechins inhibited most CYPs (21,49,50). Alkaloids of tea are generally purine alkaloids, of which caffeine is the most abundant alkaloid in all six categories of tea (51,52). Caffeine also inhibited the activities of CYPs, especially CYP1A2 (53). Tea extract also contains a considerable amount of amino acids, of which theanine is a nonproteinic amino acid special to tea. Theanine alone did not change CYP activity directly but modulated the biodistribution or damage for liver protection (54). The chemical components of tea have great changes according to the points of the teas, place of production, character of the cultivar, manufacturing style, cultivation method, and brewing. Therefore, phenols, alkaloids and amino acids may directly or indirectly affect the activities of CYPs (51,52). The tea extract, rather than a single component, was studied to comprehensively reflect the synergistic inhibitory effect of multiple components on the activities of CYPs.

Reference:

Nowogrodzki, A., How climate change might affect tea. *Nature* **2019,** 566, (7742), S10-S11.

Satoh, T.; Fujisawa, H.; Nakamura, A.; Takahashi, N.; Watanabe, K., Inhibitory Effects of Eight Green Tea Catechins on Cytochrome P450 1A2, 2C9, 2D6, and 3A4 Activities. *J Pharm Pharm Sci* **2016,** 19, (2), 188-97.

Misaka, S.; Kawabe, K.; Onoue, S.; Werba, J. P.; Giroli, M.; Tamaki, S.; Kan, T.; Kimura, J.; Watanabe, H.; Yamada, S., Effects of green tea catechins on cytochrome P450 2B6, 2C8, 2C19, 2D6 and 3A activities in human liver and intestinal microsomes. *Drug Metab Pharmacokinet* **2013,** 28, (3), 244-9.

Muto, S.; Fujita, K.; Yamazaki, Y.; Kamataki, T., Inhibition by green tea catechins of metabolic activation of procarcinogens by human cytochrome P450. *Mutat Res* **2001,** 479, (1-2), 197-206.

Tang, G. Y.; Meng, X.; Gan, R. Y.; Zhao, C. N.; Liu, Q.; Feng, Y. B.; Li, S.; Wei, X. L.; Atanasov, A. G.; Corke, H.; Li, H. B., Health Functions and Related Molecular Mechanisms of Tea Components: An Update Review. *Int J Mol Sci* **2019,** 20, (24).

Carrillo, J. A.; Benitez, J., Clinically significant pharmacokinetic interactions between dietary caffeine and medications. *Clin Pharmacokinet* **2000,** 39, (2), 127-53.

Sadzuka, Y.; Sugiyama, T.; Nagamine, M.; Umegaki, K.; Sonobe, T., Efficacy of theanine is connected with theanine metabolism by any enzyme, not only drug

metabolizing enzymes. *Food Chem Toxicol* **2006,** 44, (2), 286-92.

Horie, H.; Kohata, K., Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. *J Chromatogr A* **2000,** 881, (1-2), 425-38.

Comment 18:

Authors should discuss the limitation of the study, for instance, the effect of the tea on the selectivity of the method, the composition of the tea was not investigated in order to check if potential CYP inhibitors were present.

Reply 18:

Thank you very much for your professional advice. We added the limitations of the study to the revised manuscript (see lines 411-417).

Changes in the text:

Our study also has limitations; for instance, regarding the effect of tea on the selectivity of the method, the composition of tea was not investigated to check if potential CYP inhibitors were present. Further studies can be carried out on the changes in the composition of the tea extract to identify the key components to relieve the burden on the liver. Moreover, subsequent studies in human liver microsomes and in vivo studies are needed to reflect the influences of tea on CYP450 enzymes under physiological conditions and provide scientific information on potential in vivo tea-drug interactions.

Comment 19:

A conclusion section is missing!

Reply 19: Thank you for your careful review. We have added the conclusion (see lines 419-428).

Changes in the text:

Conclusions

A highly sensitive LC–MS/MS method with a suitable linear range and short analysis time for the simultaneous determination of five probe drugs (MT, OMP, PNT, TOL, and T) in RLMs was established. This method was applied to assess the inhibitory effects of aqueous extracts of four types of tea on CYP450 enzymes in vitro. These results suggested that aqueous extracts of green tea have potential influences on the metabolism of drugs mediated by CYP1A2, such as PNT, theophylline, paracetamol, caffeine and imipramine. The metabolism of drugs metabolized by CYP2C6 (ibuprofen, TOL, warfarin, phenytoin and irbesartan) would be influenced by aqueous extracts of green tea and Pu'er tea. During the concomitant use of tea with these drugs, great caution should be taken.